Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Invertebrate Pathology 81 (2002) 103–113
Journal of INVERTEBRATE PATHOLOGY
www.academicpress.com

The impact of mixed infection of three species of microsporidia isolated from the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae)

Leellen F. Solter,[a,*] Joel P. Siegel,[b] Daniela K. Pilarska,[c] and M. Catherine Higgs[a]

[a] *Illinois Natural History Survey, 140 NSRC, 1101 W. Peabody Dr., Urbana, IL 61801, USA*
[b] *USDA Agricultural Research Service, Horticultural Crops Research Laboratory, SJVASC, Parlier, CA 93648, USA*
[c] *Bulgarian Academy of Sciences, Institute of Zoology, 1 Tsar Osvoboditel Blvd., 1000 Sofia, Bulgaria*

Received 4 June 2002; accepted 23 September 2002

Abstract

The outcome of mixed infection by three species of microsporidia in the genera *Endoreticulatus*, *Nosema*, and *Vairimorpha*, isolated from different populations of *Lymantria dispar* in Bulgaria, was evaluated in the laboratory. All possible combinations of two species were administered either simultaneously or sequentially to larvae, and mortality, duration of development, and larval weight at 20 days post-infection (simultaneous inoculation) or 23 days post-infection (sequential inoculation) were chosen as the outcome variables. Larvae were also dissected and the presence of each species of microsporidia and the tissues infected were recorded for each treatment. Effects of infection were dependent on both host sex and the type of exposure. Infected larvae were more likely to die than uninfected larvae, but there were no differences in mortality between single and mixed infections. Addition of *Endoreticulatus* to infections of *Nosema* or *Vairimorpha* significantly increased duration of development to the fourth ecdysis; this effect was additive. Addition of *Nosema* or *Vairimorpha* to an existing infection had no such effect. When *Nosema* was administered simultaneously with *Endoreticulatus* or *Vairimorpha*, infected larvae weighed more than larvae that had single infections with either pathogen. *Nosema* was displaced from the silk glands by *Vairimorpha* and *Nosema* suppressed octospore formation by *Vairimorpha* in fat body. The histological evidence combined with the data on larval weight supports the hypothesis that competition occurred in mixed infections.

Keywords: *Endoreticulatus* sp.; *Nosema* sp.; *Vairimorpha* sp.; Mixed species infection; Competition

1. Introduction

At least 11 different isolates of microsporidia with varying biological and morphological characteristics have been recovered from 20 European populations of the gypsy moth, *Lymantria dispar* L., in Central and Western Europe (Austria, Bulgaria, Czech Republic, Germany, Hungary, Poland, Portugal, Romania, and Slovakia) since 1985 (Maddox et al., 1999; McManus et al., 1989). Seven species in three genera are currently described (Maddox et al., 1999; Sprague, 1977; Weiser, 1998). Three undescribed microsporidian species belonging to the genera *Vairimorpha*, *Nosema*, and *Endoreticulatus* (Pilarska et al., 1998) were collected from Bulgarian populations of *L. dispar*, one species per site (Table 1). The sites were monitored during a 5–15-year period and dissections of over 2000 individual *L. dispar* larvae collected in 1997 and 1998 confirmed this pattern of occurrence (Solter et al., 2000).

The Bulgarian isolate of *Vairimorpha* sp., an octospore-producing fat body parasite, is genetically identical (ssrDNA sequence) to isolates from the Czech Republic (GenBank Accession No. AF033315) (Baker et al., 1994; Solter et al., 2000) and Slovakia (Vossbrinck, personal communication). The *Nosema* species (GenBank Accession No. AF141129), which infects the larval silk glands and fat body tissues, is closely related, possibly conspecific, to other isolates recovered from gypsy moth populations in Central Europe, including *Nosema portugal* (GenBank Accession No. AF033316) (Maddox et al., 1999; Solter et al., 2000). The *Endoreticulatus*

* Corresponding author. Fax: 1-217-333-4949.
E-mail address: l-solter@uiuc.edu (L.F. Solter).

0022-2011/02/$ - see front matter © 2002 Elsevier Science (USA). All rights reserved.
PII: S0022-2011(02)00155-6

Table 1
Collection sites for *Lymantria dispar* microsporidia in Bulgaria

Site	Site location and elevation (m)	No. of years surveyed 1984–2001	Microsporidia from *L. dispar*
Asenovgrad	Forty springs reservoir 25 km SSE Plovdiv; 300 m	5	*Endoreticulatus* sp.
Levishte	70 km N Sofia, Rt. 16; 800 m	5	*Nosema* sp.
Rupite	13 km SSW Sandanski; 200 m	13	*Vairimorpha* sp.

species develops only in the midgut tissues of the larval host and is morphologically and biologically distinct from the *Vairimorpha* and *Nosema* species, which are more closely related (Baker et al., 1997).

It is uncommon to find more than one microsporidian species infecting an individual host or in contiguous terrestrial host populations (Weiser, 1969), although a number of insect hosts are infected by several microsporidian species in different areas of their range (Briano et al., 1995; McManus et al., 1989; Nordin et al., 1972; Pilarska et al., 1998; Weiser, 1964; Wilson, 1975). Reports of mixed infections exist (Pilarska et al., 1998, 2001; Smirnoff, 1965; Wilson and Burke, 1978; Zelinskaya, 1980) but often reports described what are now believed or known to represent various spore types produced by a single species (Maddox and Sprenkel, 1978; Pilley, 1976; Weiser, 1957, 1969, 1988; Weiser and Novotny, 1987). In the case of the gypsy moth, two species, *Endoreticulatus* sp. and *Nosema sp.*, were recovered from one site in Hungary. The paucity of mixed infections in field host populations may have implications regarding release of multiple microsporidian species for purposes of biological control of insect pests.

The purpose of this study was to evaluate the interactions among three species of microsporidia isolated from *L. dispar* in Bulgaria in order to gain insight into factors that affect successful establishment of pathogens as biological control agents. Competition was assessed by evaluating the ability of one species to exclude another from host tissues, differential mortality resulting from mixed infections, and differential larval duration as well as weight gain in mixed infections. In separate laboratory experiments, the pathogens were administered simultaneously or sequentially, following the procedures of Bauer et al. (1998), who studied interactions between microsporidia and nuclear polyhedrosis viruses. Our experiments also assessed the impact of single and double dosages of a single species of microsporidium as well as the effect of temporal variation in administration of the inoculum.

2. Materials and methods

2.1. Lymantria dispar host

Lymantria dispar (strain = New Jersey Standard) egg masses were obtained from the USDA, APHIS Laboratory at Otis Air Force Base, MA. *L. dispar* larvae were hatched and reared on meridic diet (Bell et al., 1981) in 100 ml plastic cups placed inside environmental growth chambers. Growth chamber conditions both pre- and post-treatment were 24 °C, 16 h light/8 h dark, and ≈70% RH. Larvae were reared to third instar and used in experiments within 24 h of the third instar molt. These will be referred to as "larvae" in the remainder of the paper.

2.2. Microsporidia

The three microsporidian species utilized in this study were field-collected in three sites in Bulgaria where they are enzootic in *L. dispar* populations (Table 1). New infections were produced in *L. dispar* larvae by feeding approximately 10^5 spores spread on the surface of the diet in 100 ml plastic cups, and spores were harvested from infected tissues approximately 18–20 days post-inoculation (pi) for storage in liquid nitrogen as per Solter and Maddox (1998a). For the purpose of this paper, the three species will be referred to as *Endoreticulatus*, *Nosema*, and *Vairimorpha*.

2.3. Inoculation of hosts

Small diet cubes (≈3.0 mm^3) were placed individually into wells of 24-cell plastic culture plates and inoculated with 1 μl spore suspension. Treatments were each possible combination of two species of microsporidia administered simultaneously in the first experiment, each possible combination of two species administered sequentially in a second experiment, each species administered individually in both experiments, and water controls (Table 2). Dampened filter paper was placed under the well plate lids to maintain humidity within the wells, and newly molted third instar larvae were allowed to feed for 24 h under growth chamber conditions. Larvae that consumed the entire diet cube were placed individually on fresh diet in 30-ml plastic cups with paper lids. Larvae that did not consume the entire diet cube were discarded. The first mature environmental spores occur seven days after infection, indicating one full generation of pathogen development; therefore, the second dosage in sequential inoculations was administered on day 7. The simultaneous infection experiment was replicated twice and the sequential infection experiment was replicated three times. There were no differ-

Table 2
Dosages used for simultaneous and sequential infections of third instar *Lymantria dispar* Larvae with *Endoreticulatus sp.*, *Nosema sp.*, and *Vairimorpha* sp.

Species I (spores/inoculum)	Species II (spores/inoculum)
Experiment 1: Simultaneous infections; inoculated day 1	
Nosema sp. (5×10^3)	*Endoreticulatus* sp. (5×10^3)
Nosema sp. (5×10^3)	*Vairimorpha* sp. (5×10^3)
Vairimorpha sp. (5×10^3)	*Endoreticulatus* sp. (5×10^3)
Nosema sp. (1×10^4)	NA[a]
Nosema sp. (5×10^3)	NA
Endoreticulatus sp. (1×10^4)	NA
Endoreticulatus sp. (5×10^3)	NA
Vairimorpha sp. (1×10^4)	NA
Vairimorpha sp. (5×10^3)	NA
Water	NA
Species I: inoculated day 1	Species II: inoculated day 7
Experiment 2 : Sequential infections; each dosage = 2.5×10^3	
Endoreticulatus sp.	*Endoreticulatus* sp.
Endoreticulatus sp.	Water
Water	*Endoreticulatus* sp.
Nosema sp.	*Nosema* sp.
Nosema sp.	Water
Water	*Nosema* sp.
Vairimorpha sp.	*Vairimorpha* sp.
Vairimorpha sp.	Water
Water	*Vairimorpha* sp.
Nosema sp.	*Endoreticulatus* sp.
Nosema sp.	*Vairimorpha* sp.
Endoreticulatus sp.	*Nosema* sp.
Endoreticulatus sp.	*Vairimorpha* sp.
Vairimorpha sp.	*Nosema* sp.
Vairimorpha sp.	*Endoreticulatus* sp.
Water	Water

[a] NA, not applicable.

ences between the replicates within an experiment and the data were pooled for statistical analysis.

2.4. Treatments

The dosages used in these experiments (Table 2) were chosen based on previous experimental protocols (Solter and Maddox, 1998b) to maximize competition and minimize mortality (unpublished). All inoculated larvae became infected and the infections were usually chronic, requiring more than 20 days to death or successful pupation. Two dosages were used as treatments in the single species infections to eliminate the possibility that larvae receiving the lower dosage would weigh more because they received less inoculum. There were no significant differences in larval weight or mortality between the two dosages used in the single species infections and the data were pooled for the final analysis. Mortality rates were assessed in the first set of experiments and additional baseline bioassays were performed for the three species (dosages of 10^2, 10^3, 10^4, and 10^5 spores). Based on these data, dosages for the sequential study were reduced for each inoculum of a single pathogen species. The final dosage (5×10^3 spores) is above the LD_{50} for *Nosema* sp. and *Vairimorpha* sp. but below the LD_{50} for *Endoreticulatus* sp. In the treatments where one dosage was a water control, the final dosage was 2.5×10^3 spores.

At death, adult emergence, or at a predetermined time pi (20 or 23 days), larvae were frozen for dissection. A total of 532 larvae were examined in the simultaneous infection experiment and 935 larvae were examined in the sequential infection experiment. Infection was confirmed by dissection of each individual and microscopic examination under phase contrast illumination (400×) of fresh smears of target tissues. Because later stadia female larvae are larger than male larvae (Leonard, 1981), sex was recorded for each individual based on pupal and adult morphology or examination of the larval gonads with a dissection microscope at a magnification of 25–50×.

2.5. Data collection and statistical analysis

Mortality through pupation was recorded daily for all treated and control larvae. The increased likelihood of mortality for different treatments was assessed using relative risk, a statistic employed in epidemiology (Kelsey et al., 1986). Comparisons were made between treated groups and the control, single vs. mixed infections, and single vs. double infections with the same pathogen.

The length of development until the fifth instar was recorded for all larvae and differences among treatments were analyzed as a two-way analysis of variance (ANOVA) employing SuperANOVA version 1.11 (Abacus Concepts, Berkeley, CA). When a significant interaction between treatment and sex was found, the data were split by sex and analyzed using one-way ANOVA. Post hoc comparisons of treatments were made using Fisher's protected least significant difference (lsd) test (Steel and Torrie, 1980). The interaction between pathogens was considered additive when the duration of development for mixed infections exceeded the length of development for single infections with either pathogen.

Larval weights were recorded every three days beginning four days pi. The outcome variable for simultaneous infections was larval weight on day 20, chosen because the control group had entered the prepupal stage. For sequential infections, individuals were reared until death or adult emergence and the outcome variable was larval weight on day 23. Use of this later date did not result in increased larval weight compared to day 20. The independent variables in both experiments were treatment and sex. The weight data in both experiments were analyzed with the statistical methodology and software mentioned above. The analyses of the differences between a single and double dosage of the same pathogen and the difference between a single dosage of a

pathogen at two time intervals did not utilize data from the controls. Data for single and double doses of the same pathogen were pooled when comparing mixed and single infections.

Orthogonal contrasts of both grouped and paired means utilizing multiple regression/correlation (mrc) analysis (Cohen and Cohen, 1983) were conducted by sex for the weight data in both experiments. Orthogonal contrasts are uncorrelated and when the data are normally distributed the contrasts are statistically independent of one another. This technique enabled us to group treatment means and contrast them to both single and grouped treatments and also eliminated numerous post hoc comparisons, thereby reducing the likelihood of Type I errors. For simultaneous infections, the mrc analysis consisted of the following six contrasts: control vs. all other treatments; single species vs. mixed infections; single infection with *Nosema* vs. pooled means for single infection with *Endoreticulatus* and *Vairimorpha*; two *Nosema* mixes vs. *Endoreticulatus* + *Vairimorpha*; *Nosema* + *Endoreticulatus* vs. *Nosema* + *Vairimorpha*; and single infection with *Endoreticulatus* vs. single infection with *Vairimorpha*. The following mrc analysis was conducted for the sequential infections: pooled single and sequential exposure vs. control; single exposure vs. sequential exposure; sequential exposures sharing a pathogen species contrasted (for example, *Nosema* + *Endoreticulatus* vs. *Nosema* + *Vairimorpha*). In this analysis, the data for single and double exposure to the same pathogen were pooled, with the exception of females exposed to *Vairimorpha* (only larvae infected on day 1 were used). Means are reported in all experiments ± SD.

3. Results

Endoreticulatus was not excluded from midgut tissues by either *Nosema* or *Vairimorpha* and there were no observable differences between single and mixed *Endoreticulatus* infections. Single infections with *Vairimorpha* produced hypertrophic fat body tissues containing both mature diplokaryotic environmental spores and monokaryotic octospores. The silk glands, however, appeared much like the glands of uninfected larvae and did not develop hypertrophies (Figs. 1A and B). Although primary spores (autoinfective spore forms) were observed in silk glands, no mature environmental *Vairimorpha* spores were found in silk gland tissues ($n = 88$). In contrast, the silk glands of larvae that had single infections with *Nosema*, were extensively hypertrophic (79/80) and filled with mature diplokaryotic spores (Fig. 1C). The fat body tissues were also heavily populated with diplokaryotic spores in late stage infections (≥15 days pi).

In mixed infections, silk glands of larvae that were simultaneously inoculated with *Nosema* and *Vairimorpha* developed fewer hypertrophic areas with mature *Nosema* spores (Fig. 1D). Primary spores were the predominant

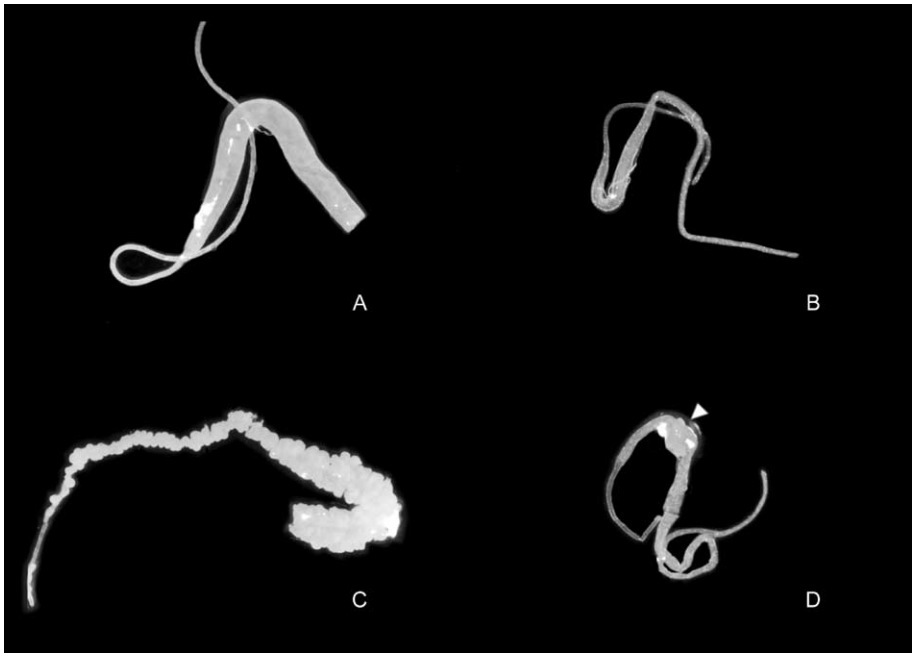

Fig. 1. Silk glands of *Lymantria dispar* infected with two species of microsporidia, *Nosema* sp. and *Vairimorpha* sp. (A) Healthy silk gland; (B) silk gland infected with *Vairimorpha* sp., only primary spores are found; (C) silk gland infected with *Nosema* sp., cells are hypertrophied and filled with environmental spores; (D) silk gland infected with both *Nosema* sp. and *Vairimorpha* sp. One area shows typical hypertrophy caused by *Nosema* sp. (arrow). Total length of gland in (A) is 2 cm.

spore forms observed ($n = 77$). One larva was the exception with silk glands that were completely hypertrophied and filled with mature environmental spores as is typical of single *Nosema* infections. When larvae were infected sequentially with *Nosema* followed by *Vairimorpha*, the silk glands followed the pattern observed in single *Nosema* infections (hypertrophic and filled with diplokaryotic spores, $n = 48$). Silk glands of larvae infected sequentially with *Vairimorpha* followed by *Nosema* contained only primary spores, typical of *Vairimorpha* infection ($n = 14$; 32 larvae died before tissues could be individually observed). In single *Vairimorpha* infections, octospores were present in fat body tissues but in larvae infected sequentially with *Nosema* followed by *Vairimorpha*, octospores were rarely observed in the fat body of most larvae by day 16 pi. Diplokaryotic spores of the two species are indistinguishable, so it was not possible to determine whether *Vairimorpha* was excluded from the fat body tissues or if only octospore formation was impaired. Octospores were always present in the fat body tissues of larvae infected sequentially with *Vairimorpha* followed by *Nosema*.

3.1. Impact of simultaneous infection on larval survival, duration, and weight

Infection rates were 100% with the exception of single infection with *Endoreticulatus* (98%). A total of 82/84 control larvae survived to 21 days pi compared to 424/478 infected larvae, 97.6 ± 15.3 and $88.7 \pm 31.7\%$ survival, respectively. Infected larvae had a relative risk of 4.75 for mortality, indicating that they were 4.75 times more likely to die than uninfected larvae ($\chi^2 = 5.35$, $0.025 > P > 0.01$). There were no differences in mortality between single and mixed infections.

Duration of development was both treatment and sex dependent (Two-way ANOVA Model F value $= 23.5$, $P = 0.0001$), Table 3. Males infected with *Vairimorpha*, either alone or in combination with a second pathogen, took longer to reach the fourth ecdysis than female larvae in the same treatments (treatment × sex interaction F value $= 2.5$, $P = 0.02$). The length of development depended on treatment for each sex (One-way ANOVA Model F value $= 29.5$, $P = 0.0001$, Model F value $= 19.9$, $P < 0.001$, males and females, respectively). Among males, the single *Nosema* infection group took almost one day longer to develop ($P < 0.005$) than the control, single infection with *Endoreticulatus*, and simultaneous infection with *Nosema* + *Endoreticulatus* treatments. All treatments with *Vairimorpha* took the longest to develop ($P = 0.001$). Among females, only treatments with *Vairimorpha* delayed development ($P = 0.001$).

Larval weight was affected by treatment and sex and overall, females were heavier (Two-way ANOVA Model F value $= 86.3$, $P = 0.0001$). For each sex, the treatments had a differing impact on weight (One-way ANOVA Model F value $= 47.8$, $P = 0.0001$, Model F value $= 88.2$, $P = 0.0001$, males and females, respectively). The mean weight values for all treatments are summarized in Table 3. There were no weight differences between the overall means of single and mixed infections. Among males, the control group weighed more than the overall mean of the pathogen treatments ($P < 0.0001$), but there was no weight difference between the control group and the single infection with *Nosema* treatment. Larvae infected with *Nosema* were heavier than the combined mean for the single infections with *Endoreticulatus* and *Vairimorpha* ($P < 0.0001$). Males with single *Endoreticulatus* infections weighed more than males with single *Vairimorpha* infections ($P = 0.002$). When mixed infections were contrasted, males infected with *Nosema* were heavier than males infected with the other pathogens ($P < 0.0001$). When the two *Nosema* mixed infections were compared, males infected with *Nosema* + *Endoreticulatus* weighed more than males infected with

Table 3
Simultaneous inoculation: Duration of *Lymantria dispar* development and larval weight (g) ± SD on day 20 pi

Treatment[a]	Sex	*N*	Days to fifth instar	Weight (g)
Control	Male	39	10.1 ± 0.6	0.696 ± 0.066
Nosema	Male	38	11.2 ± 1.8	0.652 ± 0.204
Endoreticulatus	Male	34	10.3 ± 0.7	0.388 ± 0.099
Vairimorpha	Male	38	14.5 ± 2.3	0.274 ± 0.140
Nosema + *Endoreticulatus*	Male	40	10.3 ± 1.1	0.572 ± 0.146
Nosema + *Vairimorpha*	Male	39	13.3 ± 2.5	0.416 ± 0.191
Endoreticulatus + *Vairimorpha*	Male	34	12.0 ± 1.8	0.295 ± 0.157
Control	Female	43	10.4 ± 0.9	1.798 ± 0.526
Nosema	Female	40	10.6 ± 1.2	0.773 ± 0.364
Endoreticulatus	Female	44	10.3 ± 0.6	0.546 ± 0.270
Vairimorpha	Female	39	12.9 ± 2.3	0.415 ± 0.173
Nosema + *Endoreticulatus*	Female	33	10.3 ± 0.9	0.760 ± 0.427
Nosema + *Vairimorpha*	Female	33	12.6 ± 2.5	0.528 ± 0.230
Endoreticulatus + *Vairimorpha*	Female	38	12.0 ± 1.6	0.374 ± 0.200

[a] Third instar *L. dispar* larvae treated with one microsporidian species or two simultaneously administered microsporidian species on day 1 after molt to third instar.

Nosema + *Vairimorpha* ($P < 0.0001$). Among females, the control group was heavier than the overall mean of the pathogen treatments and the female control group weighed more than the single infection with *Nosema* group ($P < 0.0001$). Females infected solely with *Vairimorpha* weighed less than females infected singly with *Nosema* or *Endoreticulatus*, and also weighed less than the combined mean of these two treatments ($P = 0.0002$). Females with single *Nosema* infections weighed more than females with single *Endoreticulatus* infections ($P = 0.002$). When mixed infections were contrasted, larvae from treatments that included *Vairimorpha* weighed less than larvae from treatments without *Vairimorpha* ($P < 0.0001$). There was no weight difference between females from the two *Vairimorpha* mixed infections.

3.2. Effect of single and double dosages of the same species on larval survival, duration, and weight

There were no differences in mortality between single and double dosages of the same microsporidian species. There were significant differences in duration of development among treatments (Model F value = 6.2, $P = 0.0001$) but the only difference between single and double doses of the same species occurred in larvae infected with *Vairimorpha* (Tables 4 and 5). Administration of a second dose of *Vairimorpha* resulted in an almost two-day delay in development ($P < 0.005$). When weight differences were analyzed, the only group affected by a double dose were females infected with *Vairimorpha*. Females that received a second dose were 37% lighter ($P = 0.0001$).

3.3. Effect on larval survival, duration, and weight due to temporal difference in exposure to a single species

There were no differences in mortality observed among treatments. There were no differences in duration of development among early infection treatments or among late infection treatments, but significant differences occurred when early and late treatments were compared (Model F value = 4.3, $P < 0.005$). Overall, larvae infected early took one day longer to develop than the control group, and for *Nosema* and *Vairimorpha*, larvae infected late reached the fourth ecdysis almost one day sooner than larvae infected early ($0.05 > P > 0.01$; $P < 0.005$, respectively).

Weight differed between early and late infection groups and also differed by sex, with females being heavier (Model F value = 4.6, $P = 0.0001$). Among males, late infection larvae were heavier than early infection larvae (14.6–36.9%) for all three pathogens, Table 4. Females varied in their response, Table 5. The only significant difference, 32.6% ($P = 0.02$), occurred among *Nosema* early and late infection treatments. When the data for the females that received two dosages of *Endoreticulatus* were combined with the early infection data to increase sample size, *Endoreticulatus* late infection females were 30.7% heavier ($P = 0.01$).

3.4. Effect of sequential exposure to two species of microsporidia on larval survival, duration, and weight

There were no differences in mortality among treatments. When duration of development was compared,

Table 4
Sequential inoculation: duration of male *Lymantria dispar* development and larval weight (g) ± SD on day 23 pi

Treatment[a]	*N*	Days to fifth instar	Mean (g)
Control	28	12.3 ± 0.9	0.606 ± 0.116
Nosema + water	31	13.5 ± 1.1	0.615 ± 0.172
Nosema + *Nosema*	27	13.5 ± 1.1	0.651 ± 0.261
Nosema + *Endoreticulatus*	29	15.0 ± 3.0	0.475 ± 0.232
Nosema + *Vairimorpha*	15	15.3 ± 3.4	0.605 ± 0.258
Water + *Nosema*	19	12.7 ± 1.0	0.705 ± 0.146
Endoreticulatus + water	33	13.0 ± 1.3	0.441 ± 0.138
Endoreticulatus + *Endoreticulatus*	34	12.8 ± 1.2	0.438 ± 0.135
Endoreticulatus + *Nosema*	36	12.6 ± 1.1	0.466 ± 0.156
Endoreticulatus + *Vairimorpha*	35	13.2 ± 1.9	0.423 ± 0.116
Water + *Endoreticulatus*	31	12.7 ± 0.9	0.652 ± 0.129
Vairimorpha + water	21	12.9 ± 1.7	0.480 ± 0.177
Vairimorpha + *Vairimorpha*	29	14.3 ± 3.6	0.479 ± 0.212
Vairimorpha + *Nosema*	20	16.2 ± 6.2	0.446 ± 0.295
Vairimorpha + *Endoreticulatus*	19	15.0 ± 4.1	0.374 ± 0.192
Water + *Vairimorpha*	28	13.0 ± 1.1	0.657 ± 0.151

[a] *Lymantria dispar* larvae treated with one microsporidian species on day 1 after molt to third instar or on day 7 after molt, or two microsporidian species administered sequentially on day 1 and day 7.

Table 5
Sequential inoculation: duration of female *Lymantria dispar* development and larval weight (g) ± SD on day 23 pi

Treatment[a]	*N*	Days to fifth instar	Mean (g)
Control	36	11.7 ± 1.8	0.859 ± 0.331
Nosema + water	32	12.9 ± 1.2	0.601 ± 0.208
Nosema + *Nosema*	41	13.3 ± 1.3	0.644 ± 0.306
Nosema + *Endoreticulatus*	34	13.2 ± 0.9	0.596 ± 0.188
Nosema + *Vairimorpha*	36	13.1 ± 0.9	0.614 ± 0.289
Water + *Nosema*	40	12.4 ± 1.7	0.797 ± 0.381
Endoreticulatus + water	26	13.0 ± 2.0	0.625 ± 0.362
Endoreticulatus + *Endoreticulatus*	33	12.0 ± 1.4	0.604 ± 0.283
Endoreticulatus + *Nosema*	31	12.6 ± 1.6	0.453 ± 0.284
Endoreticulatus + *Vairimorpha*	27	12.6 ± 1.9	0.539 ± 0.202
Water + *Endoreticulatus*	27	11.9 ± 1.9	0.801 ± 0.382
Vairimorpha + water	28	13.7 ± 3.7	0.731 ± 0.501
Vairimorpha + *Vairimorpha*	25	16.2 ± 8.0	0.459 ± 0.273
Vairimorpha + *Nosema*	24	13.0 ± 2.7	0.702 ± 0.417
Vairimorpha + *Endoreticulatus*	28	14.3 ± 4.3	0.637 ± 0.471
Water + *Vairimorpha*	32	11.9 ± 1.9	0.670 ± 0.216

[a] *Lymantria dispar* larvae treated with one microsporidian species on day 1 after molt to third instar or on day 7 after molt, or two microsporidian species administered sequentially on day 1 and day 7.

both sex and treatment affected development (Model F value for treatment = 5.7, $P = 0.0001$, Model F value for sex = 20.7, $P = 0.0001$, treatment × sex F value = 2.3, $P > 0.05$). Overall, males took one day longer to develop than females. Among males, addition of *Endoreticulatus* to both *Nosema* and *Vairimorpha* infections significantly increased development time by 1.5–2 days ($P = 0.01$; $P = 0.001$, respectively). The duration of development of the mixed infection significantly differed from the values for single infections for all three pathogens ($P < 0.01$). Conversely, addition of *Nosema* or *Vairimorpha* had no effect on *Endoreticulatus* infection. Addition of *Vairimorpha* to an existing *Nosema* infection and *Nosema* to an existing *Vairimorpha* infection increased developmental time ($P < 0.001$, respectively) and the duration of development of the mixed infection significantly differed from the values for single infections for all three pathogens ($P < 0.005$). Females in three treatments, *Nosema*, *Vairimorpha*, and *Vairimorpha* + *Endoreticulatus*, took longer to develop than the control group. Addition of a second pathogen did not affect the duration of female development.

Larval weight depended on both treatment and sex and each sex responded differently to some treatments (treatment Model F value = 6.2, $P = 0.0001$; sex Model F value = 41.1, $P = 0.0001$; treatment × sex interaction F value = 2.2, $P = 0.005$). The mean weight values for males are summarized in Table 4 and for females in Table 5. Males infected with *Nosema* weighed the same as controls while *Nosema*-infected females weighed less than the controls. Addition of *Endoreticulatus* to existing male *Nosema* infections significantly decreased larval weight by 22.8% ($P = 0.04$). This effect was additive relative to *Nosema*, because the mixed infection treatment weighed the same as the single infection with *Endoreticulatus*. Addition of *Vairimorpha* to male larvae infected with *Nosema* had no such effect. Addition of either *Endoreticulatus* or *Vairimorpha* to females initially infected with *Nosema* had no effect on weight. Males and females infected with *Endoreticulatus* weighed less than the controls ($P < 0.0001$). Addition of either *Nosema* or *Vairimorpha* to males infected with *Endoreticulatus* had no effect, but in females, the addition of *Nosema* reduced larval weight by 27.5% ($P = 0.03$). This effect was additive and the females in this treatment weighed less than the females with single infections of *Nosema* or *Endoreticulatus* ($P = 0.01$; $0.05 > P > 0.01$, respectively). Males infected with *Vairimorpha* weighed 20.8% less than the controls ($P = 0.0002$), but there was no difference between the weight of control and *Vairimorpha*-infected females (probability was borderline, $P = 0.06$). The female data for *Vairimorpha* conflicted with the data from the simultaneous infections. Data were quite variable, with a coefficient of variation ([standard deviation/mean] × 100) ranging from 59.4 to 73.9. This variability could account for our inability to detect differences among treatments. Addition of *Nosema* or *Endoreticulatus* to *Vairimorpha* infections did not affect weight.

4. Discussion

In these experiments, interaction among the pathogens could be additive, neutral, or competitive/antagonistic. We presumed that if two pathogens competed, one species would exclude the other from host tissues they both utilized or in some way alter the rate of

multiplication of one pathogen, which in turn would affect the rate of tissue disruption and/or host metabolism. Competition was evaluated directly by observing target tissues and indirectly by comparing developmental time and larval weight. Larval development and weight were chosen as endpoints because overemphasis of lethal effects ignores other effects of a chronic pathogen (Siegel et al., 1986; Siegel et al., 2002; Solter et al., 1990) that can determine success in the field.

Simultaneous inoculations evaluated scramble competition, since both species had equal opportunity to establish infection. Sequential treatments were more complicated and had three objectives. The main objective was to ascertain the ability of a second pathogen to become established after the first species initiated infection. Secondary objectives were to determine whether a threshold dose existed for the effect of infection on developmental time and weight, as well as the effect of temporal variation in infection on these two parameters. In both experiments, the outcome of infection was complicated by sex-based differences, such as the increased development time of infected males compared to females or the ability of single infection with *Nosema* to decrease female weight while males remained unaffected. This finding agrees with Wilson (1978) who previously reported a differential response to infection by lepidopteran microsporidia.

In simultaneous treatments, both weak additive and antagonistic effects were observed, depending on the parameter measured. The effect of co-infection by *Vairimorpha* with either *Endoreticulatus* or *Nosema* was weakly additive, because developmental times were significantly greater than for single infection with *Endoreticulatus* or *Nosema*. The weight data revealed antagonism between the pathogen species. For both sexes, the larvae from simultaneous infections with *Nosema* and either *Vairimorpha* or *Endoreticulatus* were of intermediate weight compared to the baseline values for the single species infections. *Nosema* and *Endoreticulatus* infect different tissues and, by an undetermined mechanism, *Nosema* may have moderated the effect of midgut infection by *Endoreticulatus*. In contrast, *Vairimorpha* and *Nosema* infect the same tissues and *Vairimorpha* excluded *Nosema* from the silk glands (Fig. 1). However, there was no evidence that *Vairimorpha* excluded *Nosema* from the fat body. Infection of this organ may play a larger role than silk gland infection in determining larval weight.

We were unable to observe or quantify differences in spore production between *Nosema* and *Vairimorpha* in the fat body because the diplokaryotic spores of *Nosema* and *Vairimorpha* cannot be distinguished under light microscopy or transmission electron microscopy (unpublished data). Quantifying the environmental spore production for each species would have been a more definitive way to assess the result of competition. When the interaction between *Endoreticulatus* and *Vairimorpha* was assessed with weight as the outcome variable, *Vairimorpha* had a weakly additive effect because the group with the mixed infection weighed less than the group with the single infection with *Endoreticulatus*. The weight of the mixed infection group did not differ from the weight of the single infection with *Vairimorpha* group. These data indicate that the combined infection of the midgut, silk glands, and fat body had a greater impact on weight than a midgut infection alone. One concern in analyzing the data for simultaneous infections was the possibility that they differed from single species infections because the larvae received a lower dosage of each pathogen species (Table 2). However, orthogonal comparisons found no overall difference between single species and mixed infections and additional experiments confirmed that the half dosage used in this study was sufficient to both infect larvae and produce mortality.

In sequential infection experiments, with the exception of *Vairimorpha*, a second dose of the same pathogen had no effect on mortality, developmental time, or weight. Maddox (1966) reported that there were no additional effects on hosts that ingested more than one inoculum of spores, and the data from our study concur. *Vairimorpha* was problematic in this experiment because even the outcome of single *Vairimorpha* infections differed from the simultaneous infection experiment. *Vairimorpha*-infected females weighed the same as controls and the timing of infection did not affect female weight (Table 5). *Vairimorpha*-infected males weighed less than the controls, but they were still almost twice as heavy as their single infection counterparts in the simultaneous infection experiment. We conclude that *Vairimorpha* lost virulence, possibly due to several passages through the host. Virulence differences between isolates of the same species have been documented for other microsporidia such as *Nosema carpocapsae* and *N. pyrausta* but the mechanism is unknown (Siegel et al., 1986; Siegel et al., 2002).

Larval age at inoculation affected developmental duration (time to fourth ecdysis) and weight. We assumed that larvae infected at a later date would develop faster and weigh more than larvae infected earlier. This may occur because the older larvae had greater energy reserves and/or may also be more tolerant to infection. The magnitude of the weight difference at 23 days between larvae infected early and late was both sex and pathogen species dependent. Among males, the greatest weight difference occurred with *Endoreticulatus* while in females the greatest difference in weight occurred with *Nosema*.

Analysis of the sequential infection data indicated that overall, the impact of mixed infections was greater in males than females. Among males, the impact of

Endoreticulatus infections on duration of development and weight was unaffected by the addition of a second pathogen. Conversely, addition of *Endoreticulatus* to existing infections of the other pathogen species altered these parameters. The relationship between *Nosema* and *Vairimorpha* was complicated, and addition of either pathogen to an existing infection had a synergistic effect on duration of development in males but did not affect weight. Among females, addition of a second pathogen species had no effect on duration of development or on larval weight, with one exception, *Nosema* decreased larval weight when added to existing *Endoreticulatus* infections.

We could not determine that a single species was dominant in this study, although we demonstrated antagonism between *Nosema* and *Vairimorpha*. When *Vairimorpha* was added to an existing *Nosema* infection, octospores, normally present by day 6 pi at these dosages (Henn and Solter, 2000), did not develop, and *Vairimorpha* excluded *Nosema* from silk gland tissues (Fig. 1). Our finding that no species was dominant agrees with the findings of Knell et al. (1998). They studied competition between *Bacillus thuringiensis* and a granulosis virus of the Indianmeal moth (*Plodia interpunctella*), and noted that although the pathogens altered the age structure of the host population, there was no evidence to suggest that one species could out-compete the other. In contrast, Wilson (1978) reported that *Pleistophora* (*Endoreticulatus*) *schubergi* was dominant over *Nosema fumiferanae* in the spruce budworm, *Choristoneura fumiferana* (Clem.), but this may be the exception.

Field data support the hypothesis that *Endoreticulatus* sp. can coexist with other pathogen species. Most reports of naturally occurring mixed infections of terrestrial microsporidia are of *Endoreticulatus*-type species, including the lepidopteran *Pleistophora* (*Endoreticulatus*, Brooks et al., 1988; Cali and El Garhy, 1991) with either *Nosema* or *Vairimorpha* species (Pilarska et al., 2001; Smirnoff, 1965; Wilson and Burke, 1978). Competition with *Endoreticulatus* by other species of *L. dispar* microsporidia for midgut tissue may not be intense. If production of environmental spores is the proper way to measure competition in mixed infections, the species that produces the most spores is the winner. We did not assess this in our study, however, other factors such as relative success in host-to-host transmission, longevity of spores in the environment, and response of infected hosts to various environmental stresses may be important in the outcome of competition between microsporidia for hosts. These factors, combined with competition for the same host tissues, may suggest why *Nosema* and *Vairimorpha* species have not been found to occur sympatrically in *L. dispar* populations.

Competition between introduced pathogens with each other as well as with indigenous pathogen species could seriously complicate a purposeful introduction for biological control of an insect pest. While interactions have been studied between naturally occurring pathogens where hosts are native or established (Fuxa, 1979; Jordon and Noblet, 1982; Lewis et al., 1983; Moawed et al., 1987; Wilson, 1978), the focus on the introduction of exotic pathogens has been primarily on nontarget host effects. Information on interactions among existing pathogens or between pathogens being considered for introduction is essential to predict successful establishment of an entomopathogenic biological control agent. Our study demonstrates that these interactions are complex and, in the case of *L. dispar*, depend on host sex. Previous field collections and information about competition produced in the laboratory suggest that the *L. dispar* microsporidia may compete in *L. dispar* populations. Follow-up laboratory studies on transmission may be necessary to predict the likelihood of successful establishment of microsporidia in the field, as well as help determine whether multiple species introductions are advisable.

Acknowledgments

The authors thank Dr. Leah Bauer for discussions on evaluating pathogen interactions in the gypsy moth host and gratefully acknowledge the technical assistance of Stephen Lavallee, André Corbin, Elise Coleman, Dustin Wakeman, LuCinda Patton and Ken Bach. This research was supported in part by the Illinois Natural History Survey, USDA/ARS/HCRL, the Bulgarian Academy of Sciences, Institute of Zoology, and USDA Forest Service Cooperative Agreement 23-829, the Office of Research/Illinois Agricultural Experiment Station Project No. 65-309-S265, and the State of Illinois IDNR SW RGES 1003.

References

Baker, M.D., Vossbrinck, C.R., Becnel, J.J., Maddox, J.V., 1997. Phylogenetic position of *Amblyospora* Hazard & Oldacre (Microspora: Amblyosporidae) based on small subunit rRNA data and its implication for the evolution of microsporidia. J. Euk. Microbiol. 44, 220–225.

Baker, M.D., Vossbrinck, C.R., Maddox, J.V., Undeen, A.H., 1994. Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data). J. Invertebr. Pathol. 64, 100–106.

Bauer, L.S., Miller, D.L., Maddox, J.V., McManus, M.L., 1998. Interactions between a *Nosema* sp. (Microspora: Nosematidae) and nuclear polyhedrosis virus infecting the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). J. Invertebr. Pathol. 74, 147–153.

Bell, R.A., Owens, C.D., Shapiro, M., Tardiff, J.R., 1981. Development of mass-rearing technology. In: Doane, C.C., McManus, M.L. (Eds.), The Gypsy Moth: Research Toward Integrated

Pest Management. USDA Forest Service Tech. Bull. 1584, 599–633.

Briano, J.A., Jouvenaz, D.P., Wojcik, D.P., Cordo, H.A., Patterson, R.S., 1995. Protozoan and fungal diseases in *Solenopsis richteri* and *S. quinquecuspis* (Hymenoptera: Formicidae), in Buenos Aires province, Argentina. Fla. Entomol. 78, 531–537.

Brooks, W.M., Becnel, J.J., Kennedy, G.G., 1988. Establishment of *Endoreticulatus* n.g. for *Pleistophora fidelis* (Hostounsky & Weiser, 1975) (Microsporida: Pleistophoridae) based on the ultrastructure of a microsporidium in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). J. Protozool. 35, 481–488.

Cohen, J., Cohen, P., 1983. Applied Multiple Regression/Correlation Analysis for the Behavioral Sciences, second ed. Erlbaum, Hillsdale, NJ, 545 pp.

Cali, A., El Garhy, M., 1991. Ultrastructural study of the development of *Pleistophora schubergi* Zwolfer, 1927 (Protozoa, Microsporida) in larvae of the spruce budworm, *Choristoneura fumiferana* and its subsequent taxonomic change to the genus *Endoreticulatus*. J. Protozool. 38, 271–278.

Fuxa, J.R., 1979. Interactions of the microsporidium *Vairimorpha necatrix* with a bacterium, virus, and fungus in *Heliothis zea*. J. Invertebr. Pathol. 33, 316–323.

Henn, M.W., Solter, L.F., 2000. Food utilization values of gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae) larvae infected with a microsporidium *Vairimorpha* sp. (Microsporidia: Burenellidae). J. Invertebr. Pathol. 76, 263–269.

Jordon, J.A., Noblet, R., 1982. Pathological studies of *Heliothus* infected with *Nosema helothidis* and *Vairimorpha necatrix*. J. Georgia Entomol. Soc. 17, 177–183.

Kelsey, J.L., Thompson, W.D., Evans, A.S., 1986. Methods in Observational Epidemiology. Oxford University Press, New York, 366 pp.

Knell, R.J., Begon, M., Thompson, D.J., 1998. Host–pathogen dynamics, basic reproductive rates and threshold densities. Oikos 81, 299–308.

Leonard, D.E., 1981. Bioecology of the gypsy moth. In: The Gypsy Moth: Research Toward Integrated Pest ManagementUS Dep. Agric. For. Ser. Sci. Educ. Tech. Bull. 1584.

Lewis, L.C., Cossentine, J.E., Gunnarson, R.D., 1983. Impact of two microsporidia, *Nosema pyrausta* and *Vairimorpha necatrix*, in *Nosema pyrausta* infected European corn borer (*Ostrinia nubilalis*) larvae. Can. J. Zool. 61, 915–921.

Maddox, J.V., 1966. Studies on a microsporidiosis of the armyworm *Pseudaletia unipuncta*. PhD Thesis. University of Illinois, Urbana-Champaign.

Maddox, J.V., Baker, M.D., Jeffords, M.R., Kuras, M., Linde, A., Solter, L.F., McManus, M.L., Vavra, J., Vossbrinck, C.R., 1999. *Nosema portugal*, n. sp., isolated from gypsy moths (*Lymantria dispar* L.) collected in Portugal. J. Invertebr. Pathol. 73, 1–14.

Maddox, J.V., Sprenkel, R.K., 1978. Some enigmatic microsporidia in the genus *Nosema*. Misc. Publ. Entomol. Soc. Am. 11, 65–84.

McManus, M.L., Maddox, J.V., Jeffords, M.R., Webb, R.E., 1989. Evaluation and selection of candidate microsporidia for introduction into US gypsy moth populations. In: Wallner, W.E., McManus, K.A. (Eds.), Lymantriidae: A Comparison of Features of Old and New World Tussock Moths. USDA Forest Service, NE For Exp. Stn. Gen. Tech. Rep. NE-123, 455–468.

Moawed, S.M., Saleh, M.R., Salem, S.A., 1987. Interaction of the microsporidium *Vairimorpha ephestiae* with nuclear polyhedrosis virus and their effect on Egyptian cotton leafworm, *Spodoptera littoralis*. Acta Entomol. Bohemoslov. 84, 422–430.

Nordin, G.L., Rennels, R.G., Maddox, J.V., 1972. Parasites and pathogens of the fall webworm in Illinois. Environ. Entomol. 1, 351–354.

Pilarska, D.K., Solter, L.F., Maddox, J.V., McManus, M.L., 1998. Microsporidia from gypsy moth (*Lymantria dispar* L.) populations in central and western Bulgaria. Acta Zool. Bulg. 50, 109–113.

Pilarska, D.K., Linde, A., Goertz, D., McManus, M., Solter, L., Bochev, N., Rajkova, M., 2001. First report on the distribution of microsporidian infections of browntail moth (*Euproctis chrysorrhoea* L.) populations in Bulgaria. Anz. Schädlingskinde/J. Pest Sci. 74, 37–39.

Pilley, B.M., 1976. A new genus, *Vairimorpha* (Protozoa: Microsporida), for *Nosema necatrix* Kramer 1965: pathogenicity and life cycle in *Spodoptera exempta* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 28, 177–183.

Siegel, J.P., Maddox, J.V., Ruesink, W.G., 1986. Lethal and sublethal effects of *Nosema pyrausta* on the European corn borer in central Illinois. J. Invertebr. Pathol. 48, 167–173.

Siegel, J.P., Lacey, L.A., Vossbrinck, C.R., 2002. Impact of a North American isolate of the microsporidium *Nosema carpocapsae* on the codling moth, *Cydia pomonella*. J. Invertebr. Pathol. 78, 244–250.

Smirnoff, W.A., 1965. The occurrence of *Nosema* and *Plistophora* microsporidians on *Archips cerasivoranus* (Fitch) in Quebec. Ann. Soc. Entomol. Que. 10, 121–124.

Solter, L.F., Maddox, J.V., 1998a. Physiological host specificity of microsporidia as an indicator of ecological host specificity. J. Invertebr. Pathol. 71, 207–216.

Solter, L.F., Maddox, J.V., 1998b. Timing of an early sporulation sequence of microsporidia in the genus *Vairimorpha* (Microsporidia: Burenllidae). J. Invertebr. Pathol. 72, 323–329.

Solter, L.F., Onstad, D.W., Maddox, J.V., 1990. Timing of disease-influenced processes in the life cycle of *Ostrinia nubilalis* infected with *Nosema pyrausta*. J. Invertebr. Pathol. 55, 337–341.

Solter, L.S., Pilarska, D.K., Vossbrinck, C.F., 2000. Host specificity of microsporidia pathogenic to forest Lepidoptera. Biol. Contr. 19, 48–56.

Steel, R.G.D., Torrie, J.H., 1980. Principles and Procedures of Statistics: A Biometrical Approach, second ed. McGraw-Hill, New York, 633 pp.

Sprague, V., 1977. Annotated list of species of microsporidia. In: Bulla, L.A., Cheng, T.C. (Eds.), Comparative Pathobiology II: Systematics of the Microsporidia. Plenum Press, New York.

Weiser, J., 1957. Mikrosporidien des Schwammspinners und der Goldafter. Z. Angew. Entomol. 40, 509–525.

Weiser, J., 1964. Protozoan diseases of the gipsy moth. Proc. Intl. Congr. Protozool. 1, 497–499.

Weiser, J., 1969. Immunity of insects to protozoa. In: Jackson, Herman, Singer, (Eds.), Immunity to Parasitic Animals, vol. I. Appleton-Century-Crofts.

Weiser, J., 1988. Pathological changes in the silk glands and mortality of caterpillars of *Lymantria dispar* L. caused by a *Nosema* microsporidian. Acta Entomol. Bohemoslov. 35, 257–261.

Weiser, J., 1998. Pathogens of the gypsy moth in Central Europe: host range and interactions. In: Proceedings of the Population Dynamics, Impacts, and Integrated Management of Forest Defoliating Insects, Banska Stiavnica, 18–23 August, 1996USDA Forest Service Gen. Tech. Rep. NE-247, 322–333.

Weiser, J., Novotny, J., 1987. Field application of *Nosema lymantriae* against the gypsy moth, *Lymantria dispar* L. J. Appl. Entomol. 104, 58–62.

Wilson, G.G., 1975. Occurrence of *Thelohania* sp., *Pleistophora* sp. (Microsporida: Nosematidae) in *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Can. J. Zool. 53, 1799–1802.

Wilson, G.G., 1978. Detrimental effects of feeding *Pleistophora schubergi* (Microsporida) to spruce budworm (*Choristoneura fum-*

ifernana) naturally infected with *Nosema fumiferanae*. Can. J. Zool. 56, 578–580.

Wilson, G.G., Burke, J.M., 1978. Microsporidian parasites of *Archips cerasivoranus* (Fitch) in the district of Algoma, Ontario. Proc. Entomol. Soc. Ont. 109, 84–85.

Zelinskaya, L.M., 1980. Role of microsporidia in the abundance dynamics of the gypsy moth (*Porthetria dispar*) in forest plantings along the lower Dnepr River (Ukrainian Republic, USSR). Vetinik Zool. 1, 57–62.